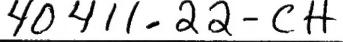


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13. ABSTRACT (Maximum 200 words) The principal objective of this project was to understand the molecular interactions between organophosphorus compounds and the enzymes that catalyze the hydrolysis of these compounds at air water-interface (Langmuir films) and in Langmuir-Blodgett films. The report contains the data on the characterization of the Langmuir films of organophosphorus acid anhydrolase (OPAA), organophosphorus acid hydrolase (OPH), and covalent bonding of the enzyme onto the substrate. Results are encouraging and the studied enzymes form stable monolayers at the interface and can be transferred on to the solid substrate. Film thickness and topography were characterized. We are trying to covalently link the enzyme molecules on to the fiberoptic cable and investigate the fluorescence.			
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Sincerely,
Roger M. Leblanc

(1) LIST OF MANUSCRIPTS submitted or published under ARO sponsorship during this reporting period, INCLUDING JOURNAL REFERENCES:

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Submitted:

- Cao, K., S.V. Mello, R.M. Leblanc, V.K. Rastogi, T.-C. Cheng and J.J. DeFrank. "A sensitive, rapid and reversible biosensor prototype for the detection of paraoxon based on the LB technique". *Colloids and Surfaces. A*.

In Press:

Published:

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- Mello, S.V., M. Mabrouki, X. Cao, R.M. Leblanc, T.-C. Cheng and J.J. DeFrank (2003). "Langmuir and Langmuir-Blodgett films of organophosphorous acid anhydrolase". *Biomacromolecules* 4, 968-973.
- Sui G., J. Orbulescu, M. Mabrouki, M. Micic, R.M. Leblanc, S. Liu, R.A. Cormier and B.A. Gregg (2003). "Self-Assembly of liquid crystal semiconductor molecules at the air-water interface". *J. Phys. Chem. B*. 106, 9335-9340.
- Zheng, Y., X. Cao, V. Konka, R.M. Andreopoulos, Si M. Pham and R.M. Leblanc (2003). "Peptidyl fluorescent chemosensors for detection of divalent copper". *Analyt. Chem.* 75, 1706-1712.
- Cao, X., G. Sui, Q. Huo and R.M. Leblanc (2002). "Langmuir and Langmuir- Blodgett films of a novel tryptophan peptide lipid". *Chem. Commun.* 8/B106 597C, 806-807.
- Cao, X., S.V. Mello, G. Sui, M. Mabrouki, R.M. Leblanc, V.K. Rastogi, T.C. Cheng and J.J. De Frank (2002). "Surface chemistry, spectroscopic and microscopic properties of organophosphorous hydrolase Langmuir and Langmuir-Blodgett films". *Langmuir* 18, 7616-7622.
- Sui, G., P. Kele, J. Orbulescu, Q. Huo and R.M. Leblanc (2002). "Synthesis of a coumarin based fluorescent amino acid". *Lett. Pept. Sci.* 8, 47-51.
- Sui, G., M. Mabrouki, Y. Ma, M. Micic and R.M. Leblanc (2002). "A structural study of amphiphilic PAMAM Poly (amido amine) dendrimers in Langmuir and Langmuir – Blodgett films". *J. Colloid Interface Sci.* 250, 364-370.
- Zheng, Y., M. Micic, S.V. Mello, M. Mabrouki, F.M. Andreopoulos, V. Konka, Si M. Pham and R.M. Leblanc (2002). "PEG-based hydrogel synthesis via the photodimerization of anthracene groups". *Macromolecules*, 35, 5228-5234.
- Zheng, Y., K. Gattas-Asfura, V. Konka and R.M. Leblanc (2002). "A dansylated peptide for the reflective detection of copper ions". *Chem. Commun.*, 2350-2351

**(2) SCIENTIFIC PERSONNEL supported by this project and
HONORS/AWARDS/DEGREES received during this reporting period:**

Dr. Roger M. Leblanc	Principal Investigator	Two months summer salary
Dr. Sarita Mello	Postdoctoral Fellow	4 months salary Worked on Langmuir, layer-by-layer films of enzymes
Guodong Sui	Graduate student	4 months salary Worked on surface chemistry and spectroscopy of OPAA and OPH

- The Society of Physical Chemists of Serbia awarded Professor Roger M. Leblanc Honorary Membership of the Society for his contribution to the development of Physical Chemistry. Presented on September 26, 2002
- Provost's Award for Scholarly Activity (In recognition for excellence in research) presented by the Provost to Roger M. Leblanc on March 5th, 2002
- Corresponding Member of the Societe Royale des Sciences de Liege, Belgique. Presented to Roger M. Leblanc on January 24, 2002

(3) Report of INVENTIONS (By TITLE ONLY):

Nil

(4) SCIENTIFIC PROGRESS AND ACCOMPLISHMENTS:

a) Background and significance:

Organophosphorus (OP) compounds constitute a major group of commercial insecticides and deadly chemical warfare agents. OP pesticides and nerve agents are known to cause acute toxic effects in animals and human beings. Their mode of action has been ascribed to their ability to inhibit acetylcholinesterase. Detection and rapid decontamination of these compounds is of prime concern. Enzymatic detection and detoxification of OP compounds are rapidly gaining importance in the decontamination studies. The enzyme-based systems will be non-toxic, non-corrosive, non-inflammable and environmentally safe. Hence our study focuses on the enzymes that are inhibited by OP compounds and enzymes that catalyze the OP compounds.

b) Specific objectives:

The main thrust of the research project was to investigate the surface chemistry of and molecular interactions between OP compounds and enzymes. We plan

- i) to characterize the Langmuir films of organophosphorus acid hydrolase (OPH) and organophosphorus acid anhydrase (OPAA) by studying their interfacial and spectroscopic properties at air/water interface,
- ii) to investigate the molecular interactions between OPH and OPAA, and the OP compounds using Polarization Modulation FTIR spectroscopy,
- iii) to characterize the topography of OPH and OPAA by scanning probe microscopies and environmental scanning electron microscopy, and
- iv) to characterize the basic features of AChE biosensor based on L-B film technology.

In order to achieve these objectives, we have conducted the experiments Langmuir films of OPAA and OPH. These enzymes were supplied by Dr. Joseph J. DeFrank, V. K. Rastogi and Dr. Tu-Chen Cheng at U. S. Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, Maryland. We have research collaboration with them and they are isolating and characterizing OPAA and OPH for our work.

c) Work with organophosphorus acid anhydrase (OPAA):

In this report we present the work on characterization of Langmuir films of OPAA on different subphases, such as phosphate, ammonium carbonate and bis-tris-propane buffers. Monolayers at the air-water interface were characterized by measuring the surface pressure and surface potential-area isotherms. *In situ* UV-Vis absorption spectra were also recorded from the Langmuir monolayers. The enzyme activity at the air-water interface was tested by the addition of diisopropylfluorophosphate (DFP) to the subphase. LB films of OPAA were transferred to mica substrates to be studied by atomic force microscopy. Finally, a one-layer LB film of OPAA labeled with a fluorescent probe, fluorescein isothiocyanate (FITC), was deposited onto a quartz slide to be tested as sensor for DFP. The clear, pronounced response and the stability of the LB film as a DFP sensor show the potential of this system as a biosensor.

Sub-phase characterization:

Phosphate and ammonium buffers:

Using KCl-free phosphate and ammonium carbonate buffers as subphase, pH 7.5 and 8.5, respectively, no reproducible results were obtained, possibly due to the solubilization of enzyme molecules in the bulk subphase. Stable monolayers were formed, otherwise, when KCl was added to the subphase. The addition of salt in the subphase was tested in order to decrease the solubility of the enzyme monolayer and promote a better spreading condition.

Bis-tris-propane buffer:

We studied the surface pressure-area isotherms for the monolayers formed on the subphases with and without KCl (Figure 1). The salt effect here is much less pronounced when compared to that with the phosphate buffer. One possible explanation is that the ionic strength of the KCl-free buffer is already sufficient for the enzyme molecules to assume an extended and stable conformation without the addition of KCl. The limiting molecular area is approximately $2800 \text{ \AA}^2 \text{ molecule}^{-1}$, indicating changes in the conformation of the enzyme molecules in comparison to the phosphate buffer. The stability of OPAA monolayer on BTP

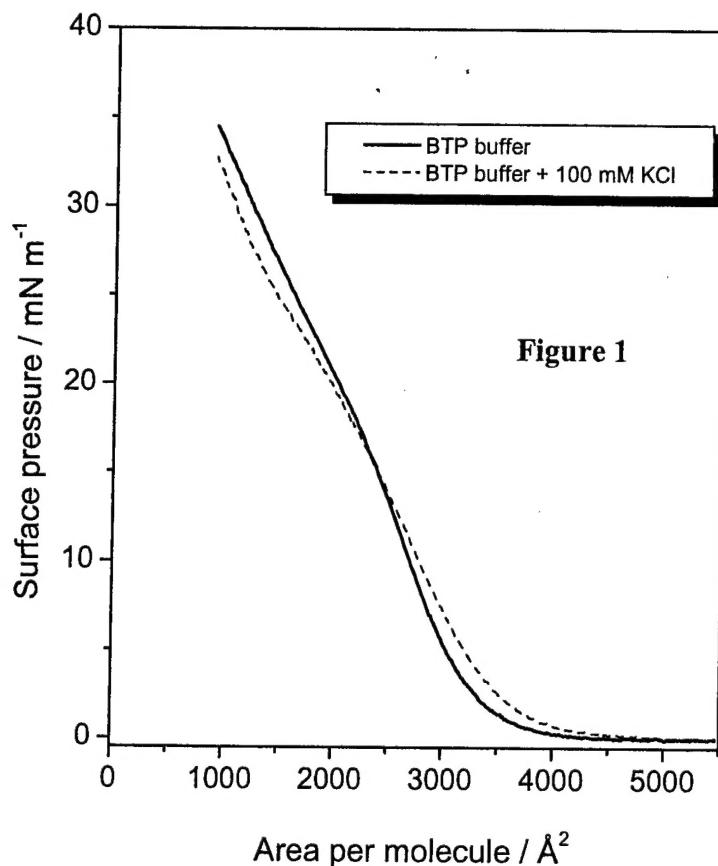


Figure 1

buffer was confirmed by measuring the decrease of area per molecule with time at constant surface pressure of 15 mN.m⁻¹. A decrease of less than 15% in the area over a period of 80 min was observed.

The surface pressure and surface potential-area isotherms were recorded simultaneously and surface potential starts increasing at a larger area per molecule (5000 Å² molecule⁻¹) than the surface pressure (3500 Å² molecule⁻¹), as seen for most Langmuir films, even though the enzyme monolayer cannot be modeled as a typical amphiphile. There is no saturation, indicating that the monolayer did not achieve any phase in which the dipole moments of its polar groups were ordered.

The measurement of UV-Vis absorption *in situ* in BTP buffer is shown in Figure 2. The absorbance increases with the surface pressure indicating the low degree of aggregation of the enzyme at air-water interface. The $\pi-\pi^*$ band at 202 nm appears at the same wavelength as for the monolayer spread on phosphate buffer. The band with its maximum at 280 nm suggests the absorption of tryptophan residues. Based on this assumption it was possible to test the intrinsic fluorescence for this enzyme at the wavelength of ~280 nm, as it will be described in the next section.

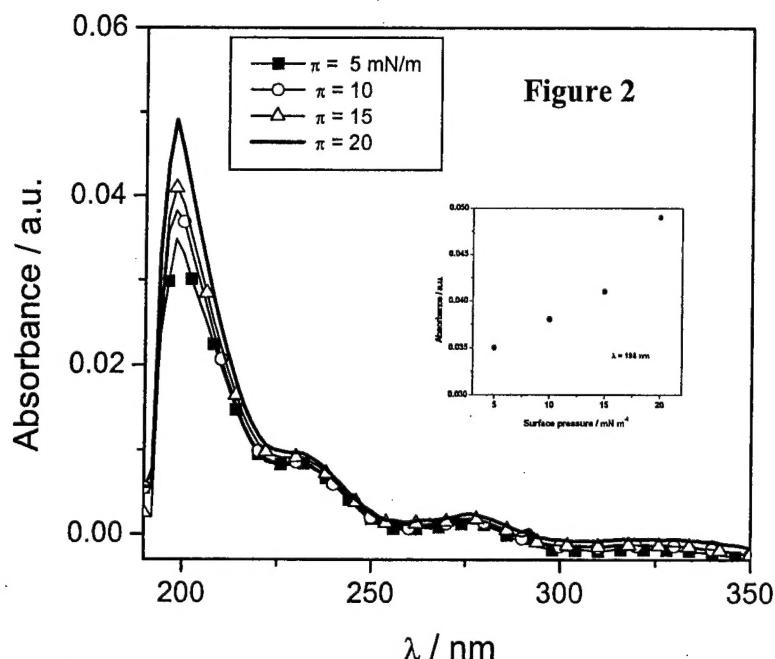
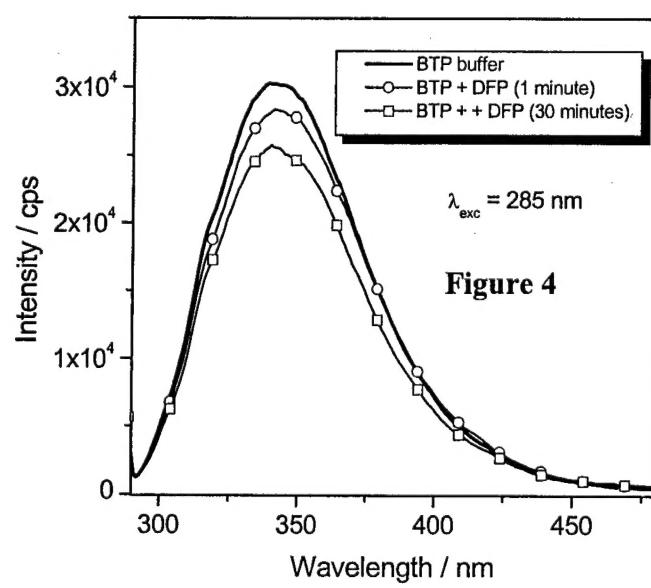
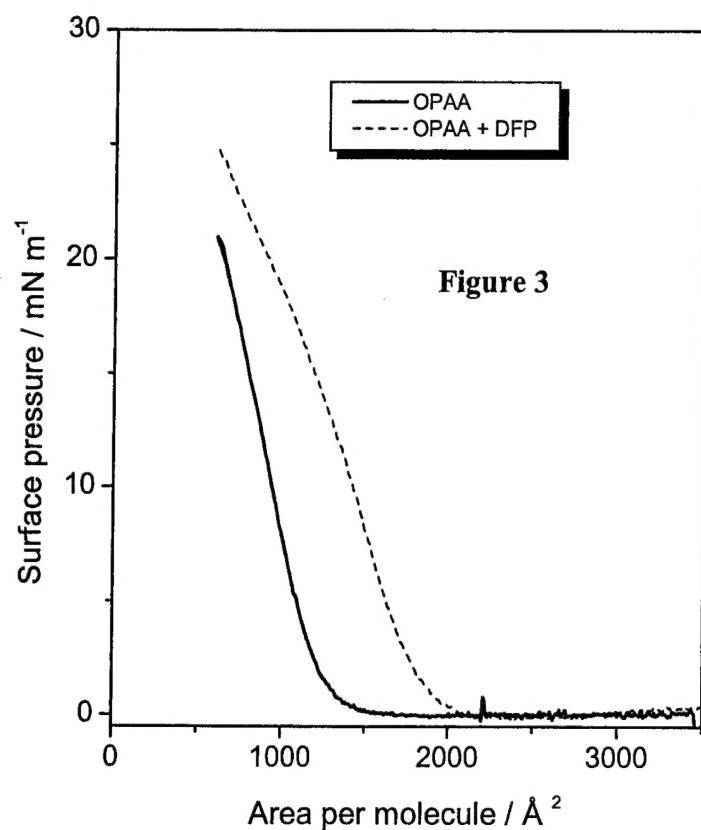


Figure 2

Activity measurements:

Langmuir monolayers:

The activity of the enzyme against paraoxon was examined by surface pressure-area isotherm and absorption spectroscopy. As expected, it was difficult to detect the low activity of OPAA against paraoxon. As known from literature, OPAA has higher selectivity against non-aromatic organophosphorus compounds such as DFP. In **Figure 3**, the effect of DFP in the subphase on surface pressure-area isotherm is presented. The mean molecular area increases significantly when DFP (10^{-4} M) is present, indicating the unfolding of enzyme molecules in the catalytic process. Since DFP is not an amphiphile molecule, we would not expect any change in the isotherm of OPAA due to an adsorption of DFP on the enzyme monolayer. This leads to the conclusion that there is reaction between the enzyme and DFP, where DFP is hydrolyzed releasing fluorine ions. The activity was also tested in solution by measuring fluorescence at ~ 348 nm when excited at 285 nm. As we can see in **Figure 4**, the addition of DFP ($\sim 10^{-4}$ M) to the enzyme solution (10^{-5} M) causes a reduction in the fluorescence intensity even after 1 minute. After 30 minutes the fluorescence intensity decreased drastically, suggesting that OPAA reacts with DFP. The fact that DFP reacts with the enzyme rules out the possibility of a simple adsorption of DFP onto the enzyme monolayer.

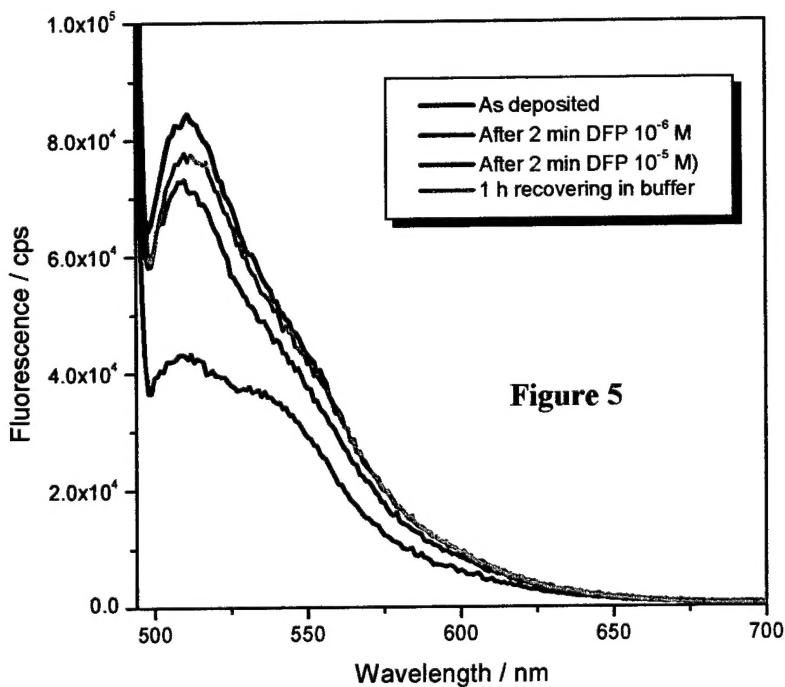


LB films of OPAA:

Langmuir monolayers were prepared in BTP buffer to obtain a stable film. OPAA monolayers were transferred to hydrophobic quartz substrates. One monolayer was deposited on to quartz slides at down strokes. The surface pressure was 20 mN/m and the deposition speed was 1.2 mm/min.

Fluorescence quenching of OPAA/FITC complex

We have tested the fluorescence quenching from the LB film of the complex OPAA/FITC in presence of DFP solution. The as-deposited LB film was tested by immersing the slide in to DFP solution at two different concentrations. The emission spectra were taken from the air dried slide. In **Figure 5** it is possible to visualize the decrease of the emission intensity of the LB film as DFP concentration was increased from 10^{-6} to 10^{-5} M (184.1 to 1841 ppb).



It is clear DFP has an effect on the fluorescence of the complex OPAA/FITC, and proves the efficiency of the system as a thin layer immobilized on a quartz surface. It is also important to notice that it was possible to recover the fluorescence intensity by immersing in buffer solution for one hour.

Figure 6 shows the quenching experiment performed in the same sample as in **Figure 5**, measured at a different condition. The slide was placed inside the cuvette with ultrapure water and DFP was added *in situ* to increase its concentration. The shape of the spectra was the same as previously observed; however, in the experiment of **Figure 6**, the quenching of fluorescence emission was observed at much lower concentration, as 10^{-7} M of DFP. The higher efficiency of the *in situ* detection over the dried sample is advantageous considering the practical use of the system as a biosensor.

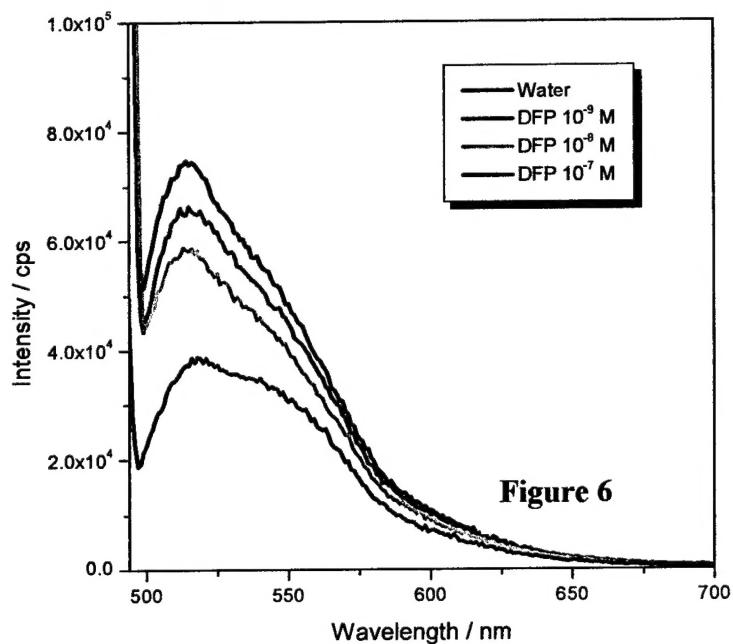


Figure 6

After the addition of the last aliquot of DFP to achieve 10^{-7} M, the slide was washed and the fluorescence was fully recovered. As a third test in the same sample, emission quenching was tested in buffer BTP, as shown in **Figure 7**. The fluorescence intensity is higher than the previous experiments reported in **Figures 5 and 6**. It is due to the fact that the enzyme activity is much more favorable in presence of the buffer although the same concentration effect was observed as in water. The total quenching was achieved with addition 10^{-7} M (18.41 ppb) of DFP.

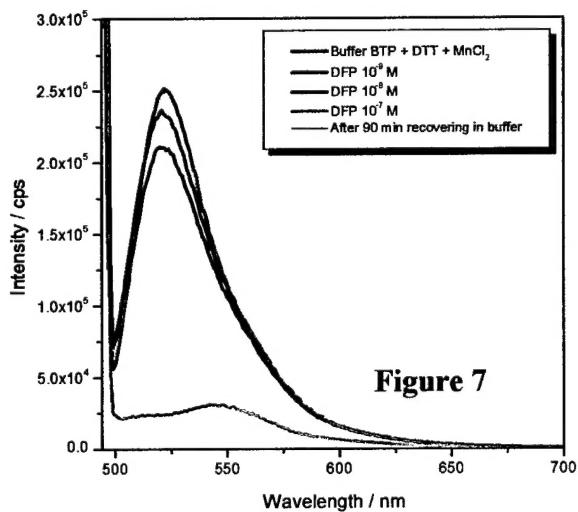


Figure 7

The full recovery of the fluorescence after the third cycle of quenching shows the good stability of this system to detect DFP in aqueous solutions. A total of five cycles was performed, showing essentially the same behavior, confirming the reproducibility of the results.

Atomic force microscopic images of LB films of OPAA:

Pure OPAA:

The OPAA monolayer was prepared on BTP buffer and 300 mM KCl as subphase and transferred onto freshly cleaved mica at 15 mN/m. The LB film was examined with TM-AFM. Images in **Figure 8** show that the shape of the enzyme molecules tends to be spherical. Size measurements indicate that OPAA can exist in the monomer (radius of a small particles is 10 nm) and dimer (big particle size is 25 nm) forms and the most abundant population is the monomer.

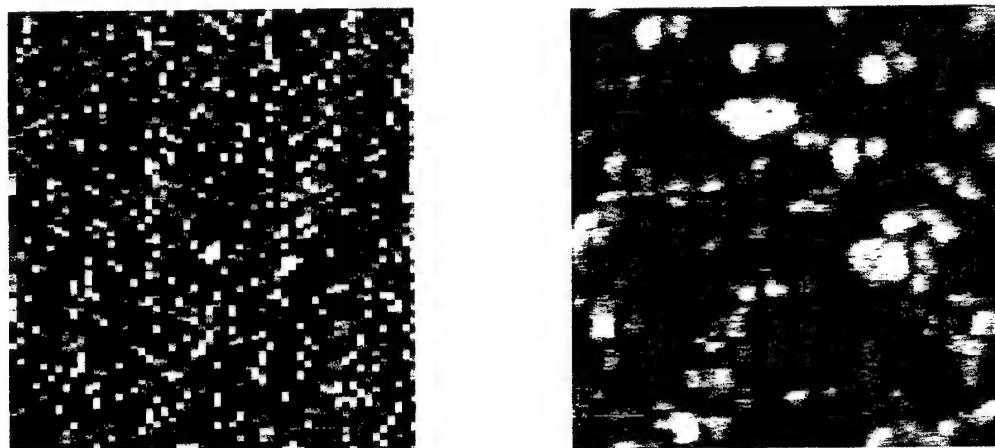


Figure 8: AFM-TM images of OPAA LB monolayer. (a 1000 x 1000 nm, b 300 x 300 nm).

Interaction of OPAA with DFP:

OPAA LB films were prepared in the subphase containing DFP 10^{-5} M. The AFM-TM images are shown in **Figure 9**. It is observed a uniform distribution of the particles that have a defined shape (ellipsoidal), although the dimensions of these particles are much larger than the OPAA dimer.

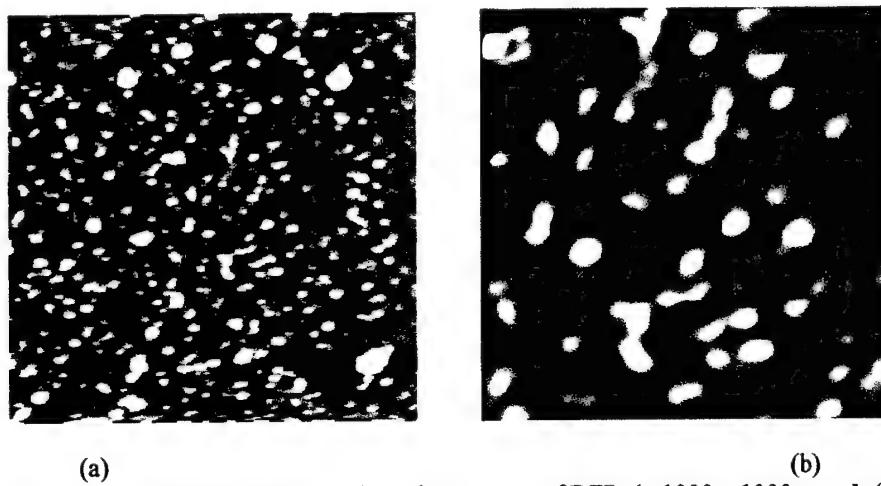


Figure 9: AFM-TM images of OPAA LB monolayer in presence of DFP. (a 1000 x 1000 nm, b (300 x 300 nm)).

In **Figure 10** the images correspond to an OPAA LB monolayer deposited from BTP buffer and dipped in DFP 10^{-5} M solution for 30 s. The sample was dried 15 min in air before performing the AFM measurements.

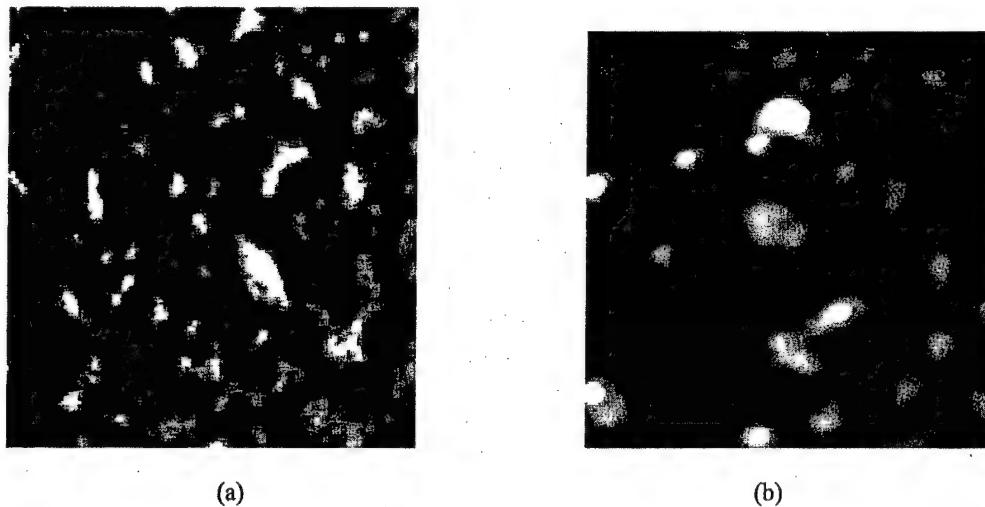


Figure 10: AFM-TM images of OPAA LB monolayer. (a 1000 x 1000 nm, b 450 x 450 nm).

At first glance, a large difference between the particles shown in **images 9a and 10a** can be noticed. In the case of **Figure 10**, two different categories of particles are seen, i.e., large ones (37x16x3 nm) and small ones (10x7x3 nm)). It should be mentioned that the large particles are similar to those in **Figure 9a**, whereas the small ones are close to the dimension of an OPAA monomer.

For a better understanding of the AFM-TM results, the progress of the hydrolysis reaction of DFP catalyzed by OPAA was followed by scanning continuously the same sample after 5 hours of the first scan. The result is shown in **Figure 11a**. The image after 1 day is shown in **Figure 11b**.

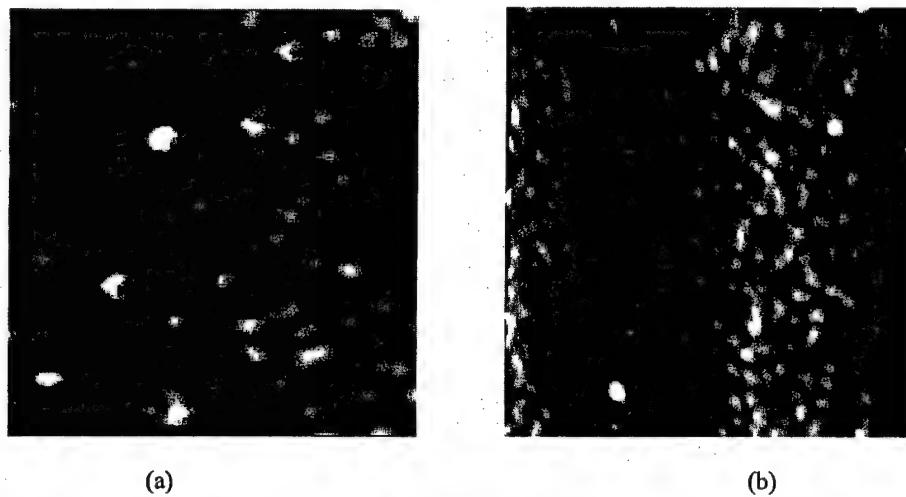


Figure 11: AFM-TM images of OPAA LB monolayer. (a 1000 x 1000 nm, b 450 x 450 nm).

This result shows that the hydrolysis reaction of DFP catalyzed by OPAA remains until all the DFP product is gone. After one day, the OPAA returns to the old form, as suggested by the third size of particle (15x6x3 nm) which appears in **Figure 11b**.

d) Work with organophosphorus hydrolase:

OPH immobilization by self-assembly technique:

As an alternative method to immobilize the enzymes on a solid support was to use the self-assembly layer-by-layer technique. It has the advantage to be experimentally simple way to built nanofilms with reasonable control of the deposition process. We started our experiments using the enzyme OPH due the availability of this material at our laboratory. *Tests with OPAA are part of our short future work.*

Brief description of the self-assembly technique:

The term self-assembly is widely used to describe the natural phenomena where molecules from a solution assemble in a preferred arrangement, as chemical or physical adsorption, as well as the formation of vesicles and micelles. Here we used the approach of electrostatic adsorption, based on interaction of charged species and also hydrogen bonding. In a simplified scheme depicted in **Figure 12**, it is described the self-assembly process of polyelectrolyte species at charged surfaces. The solid substrate is prepared to be negatively charged, as the hydrophilization of glass, for instance. The slide is then immersed in a positively charged solution, as a polycation. After the adsorption of one layer (typically 10 min), the slide is washed with a solution of the same pH as the polyelectrolyte and it is placed in the negatively charged solution, the polyanion. The same way as before, the slide was washed in a solution of the same pH and the process was repeated as many bilayers as desired. It is important to point out that after each adsorption process, the substrate is modified with the charge of the electrolyte. Since it is a macromolecule, it will let a residual charge at the surface as it folds at itself. The growing process of each layer can be followed up by UV-Vis absorption or fluorescence emission spectroscopy.

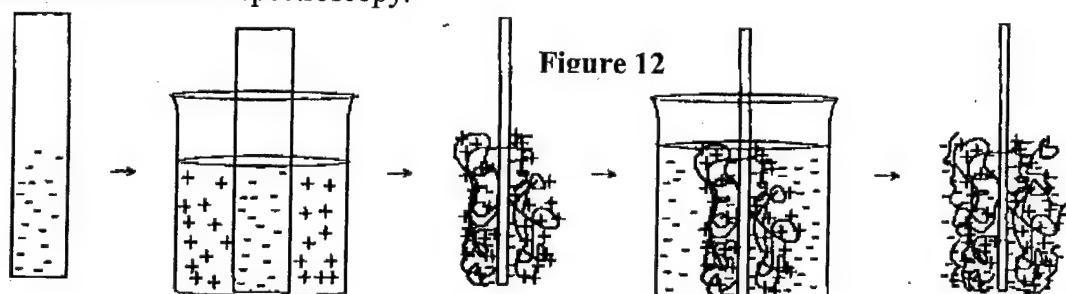


Figure 12: Schematic process of self-assembly technique.

Here, we report the immobilization of OPH on quartz substrates together with a semi conducting polymer, poly(3-thiophene acetic acid) (PTAA). We have used OPH in phosphate buffer pH 7.6, that is below its isoelectric point, which means that the enzyme molecules are positively charged, as polycations. PTAA was used as polyanion at pH 8.5 in ammonium hydroxide solution. The main idea is to immobilize the enzyme in a stable condition, as it would be in a polymeric matrix. Besides that, PTAA has fluorescence emission around 580

nm and can be used as an internal fluorescent probe for OPH. On the other hand, OPH can act as a dopant to the polythiophene molecules, opening the possibility to use this system as an opto-electric sensor in the future. In **Figure 13** it is shown the absorption spectra of a 6-bilayer OPH-PTAA film built on to a quartz slide. The band at \sim 420 nm corresponds to the PTAA layers. The increase in the UV range is due to the enzyme molecules absorption. The increases in absorbance with the number of bilayers adsorbed indicates the growing of the film as the bilayers are deposited. The high absorbance from 600 to 700 nm can have two interpretations at this moment. Either it comes from the scattering caused by the non-absorbing molecules or it can imply in the formation of a metallic band due to the free carriers created as the polythiophene is doped by OPH.

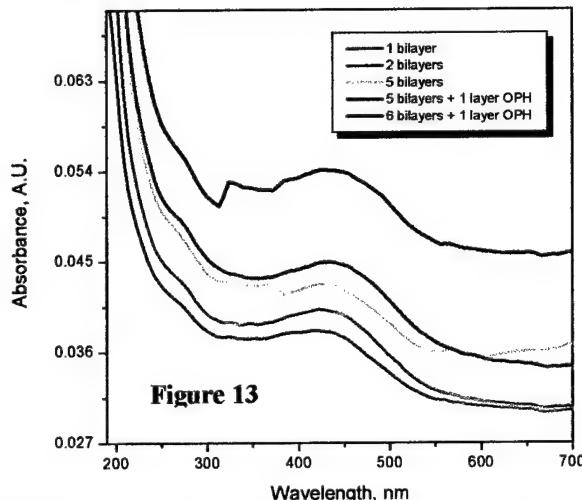


Figure 13: UV-Vis absorption of OPH-PTAA self-assembled bilayers on quartz substrate.

The deposition process was also followed by fluorescence emission of PTAA molecules. The formation of a 9-layer film was monitored after each deposition of a PTAA layer on the quartz slide. The excitation wavelength was 480 nm. The spectra are presented in **Figure 14**.

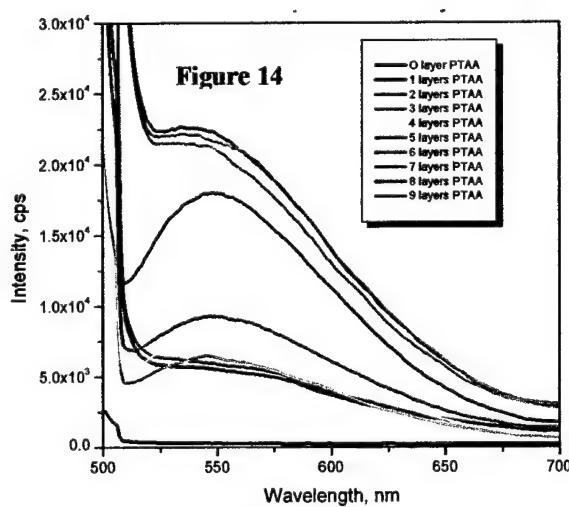


Figure 14: Fluorescence emission of OPH-PTAA self-assembled bilayers on quartz substrate.

Apparently there is saturation after the seventh layer deposition. *We are still investigating the possible causes for this result.* It was important though, to be able to follow the bilayer formation with more than one spectroscopic technique.

The activity of OPH in the self-assembled film was tested against paraoxon 10^{-9} M. It is still a preliminary result that indicates the decrease in the fluorescence intensity of OPH/PTAA complex in presence of the target molecule of OPH (Figure 15).

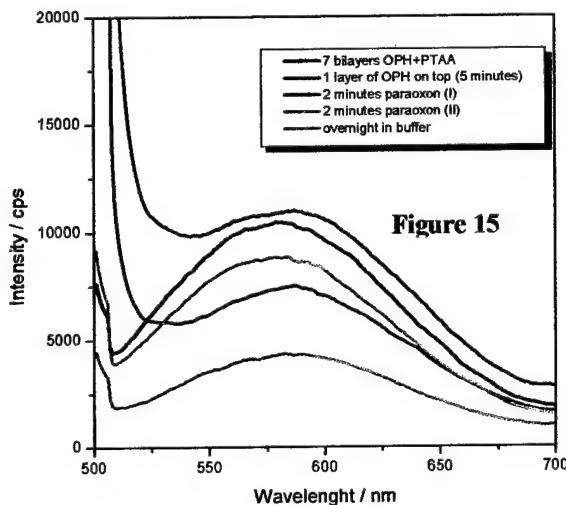


Figure 15: Fluorescence emission of OPH-PTAA self-assembled bilayers in presence of paraoxon.

After preparing 7 bilayers of OPH/PTAA, one more layer of OPH was added just before immersion of the sample into paraoxon 10^{-9} M aqueous solution. There was a little variation in the fluorescence emission, mainly below 530 nm. The intensity decreased after exposing the sample for 2 min to paraoxon in solution. After drying and washing, the sample was again placed in to paraoxon for 2 min, resulting in a lower intensity. The recovery of the fluorescence was not complete if the sample was kept overnight in buffer.

Layer-by-layer (LbL) deposition technique:

The layer-by-layer deposition technique was used to generate ultrathin films with molecular order and stability. This technique overcomes some of the disadvantages of other methods of ultrathin film assembly, *vide supra*. One major advantage of this technique is that it has great industrial application in the field of biosensors. The layer-by-layer deposition method involves the alternate adsorption of oppositely charged macromolecules such as polymers and biomacromolecules. This approach provides a simple method to develop films 5-500 nm thick that possess a high strength. Owing to its simplicity and versatility different enzymes and polyions may be assembled to fabricate an ultrathin film. These have potential applications in areas such as separation or dialysis membranes, optical devices and biosensors. Electrostatic forces, covalent bonding and to a lesser extent hydrogen bonding and hydrophobic interactions are responsible for the spontaneous assembly of these ultrathin films. The sequential adsorption of opposite charges onto the solid substrate leads to a wide range of combinations of charged species, which can be used for self-assembled ultrathin films.

Several bilayers of chitosan (CS) and poly (thiophene-3-acetic acid) (PTAA) were prepared to produce a stable supramolecular ultrathin film. CS is a high molecular weight polyglucosamine approximately 100 kDa. CS, due to strong electrostatic segment-segment repulsion, adopts an extended conformation. CS adsorbs strongly onto negatively charged surfaces and the adsorbed CS layer adopts a flat conformation that provides a stable film on which the PTAA can be adsorbed. PTAA has many important properties such as conductivity in the doped state, thermochromism, photoluminescence, fluorescence and absorption in the UV-Vis region. The first five bilayers offer a cushion of support and increased stability, as a result of strong electrostatic attraction between CS and PTAA due to the opposite charges. This allows better adsorption of OPH on this cushioning support than on the solid support such as a quartz slide. The fluorescence property of PTAA played a key role in this work as the ultrathin film was monitored using emission spectroscopy. Our methodology shows a novel way of immobilizing OPH using the alternate layer-by-layer adsorption technique. The immobilization is done under mild conditions using pH 7.3 so that the activity of OPH is not compromised. However, upon immobilization, the enzyme is stable and can be used under more harsh conditions. Layer-by-layer adsorption allows OPH to be combined with PTAA so that in the presence of paraoxon there would be a change in the optical properties of PTAA and hence detect the presence of paraoxon.

The fluorescence emission spectroscopic studies of the LbL adsorbed films:

Fluorescence of PTAA was used as a reference to monitor the growth of the CS/PTAA binary system. PTAA solution has a λ_{max} at 575 nm (Figure 16). The fluorescence spectra showed a shift toward the red region for the CS/PTAA bilayers that were adsorbed onto the quartz slide due to the formation of aggregates. The fluorescence intensity band at λ_{max} 600 nm was seen for each fluorescence spectrum that was taken. This single band at λ_{max} 600 nm corresponds to the presence of the PTAA layers. The increasing intensity of the fluorescence spectra confirms the growth of the bilayer system by the LbL deposition technique. The fluorescence intensity does not change dramatically between the fourth and fifth bilayer and at this point a homogeneous film was noted.

After building 5-bilayers of CS/PTAA, the stability of this system was tested by placing the quartz slide in pure water for 20 min. The fluorescence measurement showed no significant change in intensity. This verified that the 5-bilayer ultrathin film was sufficiently stable due to the strong electrostatic forces between the oppositely charged layers and further deposition of CS/PTAA bilayers were not necessary.

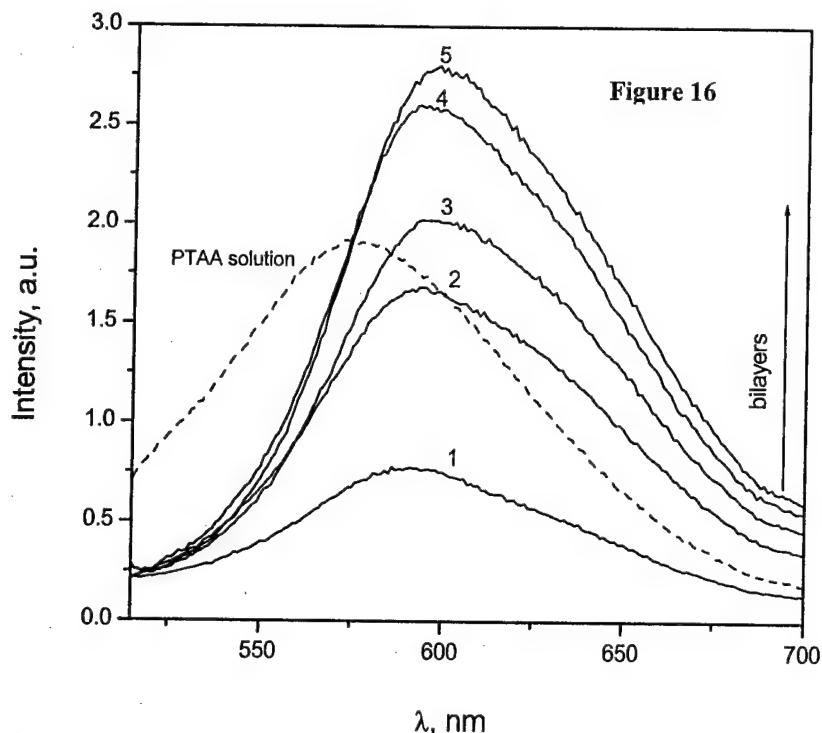


Figure 16. The fluorescence spectra of the PTAA solution (0.1mg/mL) and CS/PTAA binary system deposited onto the hydrophilic quartz slide used to confirm the CS/PTAA bilayer growing process ($\lambda_{exc} = 480$ nm): 1st CS/PTAA bilayer, 2nd, 3rd, 4th and 5th.

The OPH/PTAA bilayers continued the growth of the multilayer system and this was also followed using fluorescence spectroscopy (Figure 17). Two bilayers of OPH/PTAA were adsorbed onto the ultra-thin film of CS/PTAA that provided an independent interface for the immobilization of OPH. A layer of OPH was adsorbed as the last layer of the system in order to be able to detect paraoxon. The fluorescence spectrum was recorded after adding the last layer of OPH and this was used as the reference for further comparison of the substrate after exposure to paraoxon.

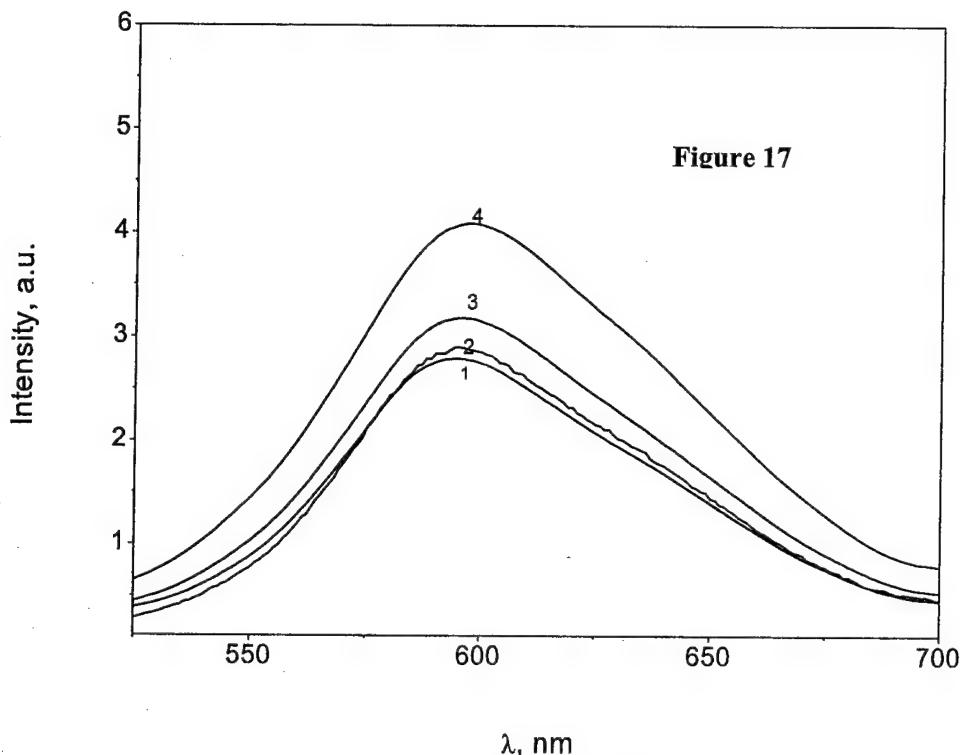


Figure 17. The fluorescence spectra of the growing OPH/PTAA bilayer system that was added on top of the 5th CS /PTAA bilayer system ($\lambda_{\text{exc}} = 480$ nm): (1) 5th. CS/PTAA bilayer before depositing OPH/PTAA, (2) 1st OPH/PTAA bilayer, (3) 2nd OPH/PTAA bilayer, (4) top OPH layer.

The sensor was placed in a small beaker containing aqueous paraoxon solution (10^{-9} M) for 1 min, removed, dried with a cool stream of air and the fluorescence was recorded. This was repeated several times with increasing concentrations of paraoxon solution. The fluorescence intensity of the sensor after exposure to paraoxon solution decreased with increasing concentrations of paraoxon solution (Figure 18). This decrease in fluorescence intensity is explained by the fact that the PTAA layer is sandwiched between two layers of OPH, i.e. one layer of OPH on both sides of the PTAA layer. Any interaction of the OPH layer with paraoxon will cause a change of conformation of the enzymes forming the layer, which causes the PTAA molecules to aggregate. Recovery of the activity of the enzyme after washing the sensor in KH_2PO_4 :NaOH buffer will favor the recovery of the enzyme conformation and as a consequence a partial disaggregation of the PTAA molecules which explains the increase of the intensity of fluorescence (Figure 18, spectra 6 and 7). The sensor could be recovered and can be reused for the detection of at least 10 samples of paraoxon.

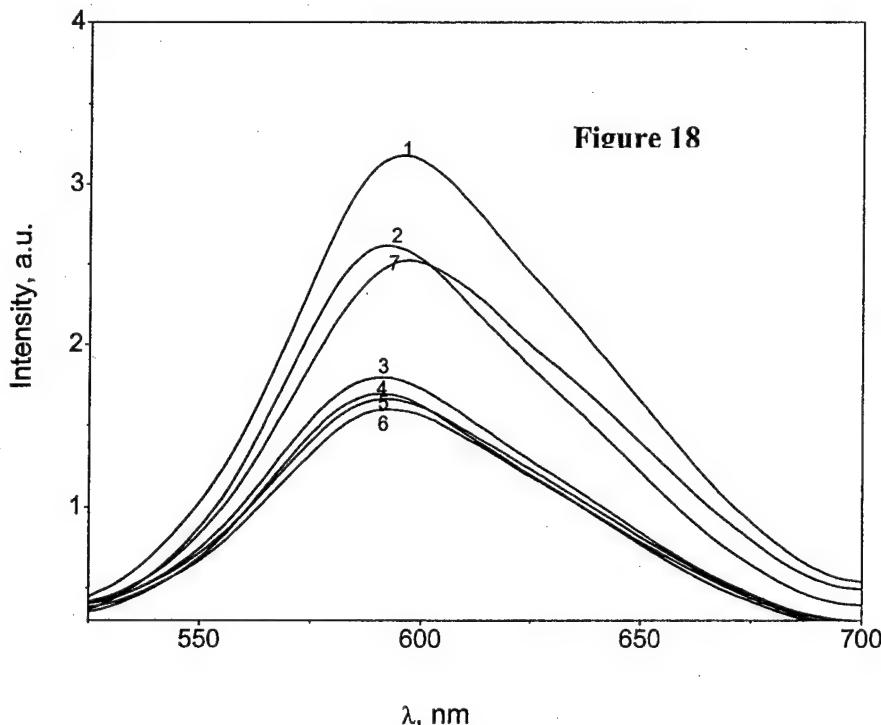


Figure 18

Figure 18. The fluorescence spectra of OPH in the presence of different concentrations of aqueous paraoxon solution ($\lambda_{\text{exc}} = 480$ nm): (1) top layer of OPH, (2) 1×10^{-9} M, (3) 1×10^{-8} M, (4) 4×10^{-8} M, (5) 8×10^{-7} M, (6) 0.5×10^{-6} M, (7) recovery of enzyme after leaving overnight in KH_2PO_4 : NaOH buffer.

A reference quartz slide was prepared on which 5-bilayers of CS/PTAA were adsorbed. The results observed are shown in **Figure 19**. The results indicate that in the absence of OPH there is marginal change in the fluorescence intensity spectra from the fifth CS/PTAA bilayers to that in the presence of paraoxon. This proves that OPH is responsible for the hydrolysis of paraoxon.

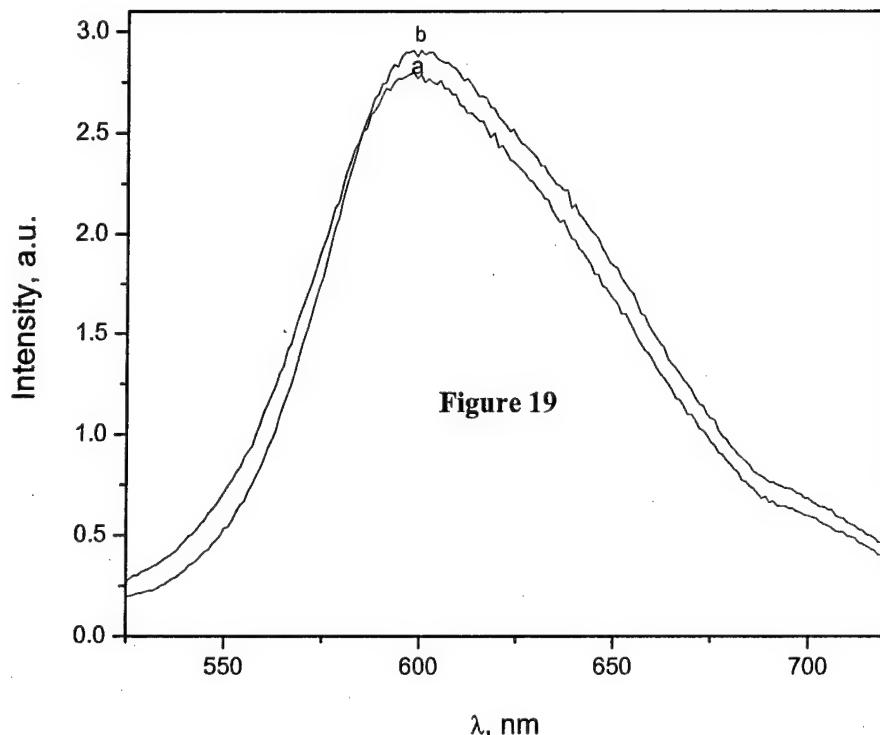


Figure 19. The fluorescence spectra of the CS/PTAA system in the absence of OPH used to prove that OPH is responsible for the paraoxon detection ($\lambda_{\text{exc}} = 480$ nm): (a) 5th bilayer CS/PTAA, (b) 10^{-6} M paraoxon solution.

The UV-Vis absorption studies for the detection of paraoxon using (LbL) adsorbed films: One method used to illustrate that the LbL adsorbed films successfully works for the detection of paraoxon is the UV-Vis absorption spectrum of p-nitrophenol (PNP). OPH hydrolyses the phosphotriester bond of paraoxon releasing the hydrolysis products, one of which is PNP ($\lambda_{\text{max}} = 400$ nm). In this experiment, an aqueous solution of paraoxon (8×10^{-7} M) was placed into a quartz cuvette and the UV-Vis spectrum was recorded. Paraoxon has a λ_{max} at 274 nm. After recording the absorption spectrum of the paraoxon solution, the sensor was placed in the quartz cuvette for different time intervals. After the initial exposure of the sensor to the paraoxon solution for 30 s, the sensor was removed and the UV-Vis absorption spectrum of the solution in the quartz cuvette was recorded. **Figure 20** shows that after exposure of the paraoxon solution in the cuvette to the immobilized OPH, the absorption spectrum showed a new λ_{max} at 400 nm (curve 2). This corresponds to the hydrolysis product PNP. The sensor was again placed in the cuvette for another 30 s so that the solution was exposed to the enzyme for 1 min. The sensor was again removed and the UV-Vis spectrum was recorded (curve 3). The procedure of exposing the paraoxon solution to the sensor was repeated several times until the total time that the paraoxon solution was exposed to the sensor was 10 min. **Figure 20** shows that the λ_{max} at 274 nm decreases with a corresponding increase in the λ_{max} at 400 nm as the exposure time increases. The isosbestic point at 292 nm at the beginning of the hydrolysis (**Figure 20**, curves 2-7), confirms that there is a transition from the paraoxon to the hydrolyzed product. The initial increase in the λ_{max} at 400 nm is steep as compared to the

absorption band after 2 min (**Figure 20**, curve 4). The rapid activity of the enzyme system is shown by the presence of the absorption band for PNP within as little as 30 s. UV-Vis spectroscopy shows that paraoxon is successfully degraded by the immobilized OPH releasing less toxic products one of which is PNP. Lower concentrations of paraoxon can also be detected but they need a longer incubation time of the sensor in the paraoxon solution in order for a measurable quantity of PNP to be produced. This sensor is very sensitive because concentrations as low as 10^{-9} M of paraoxon can be detected.

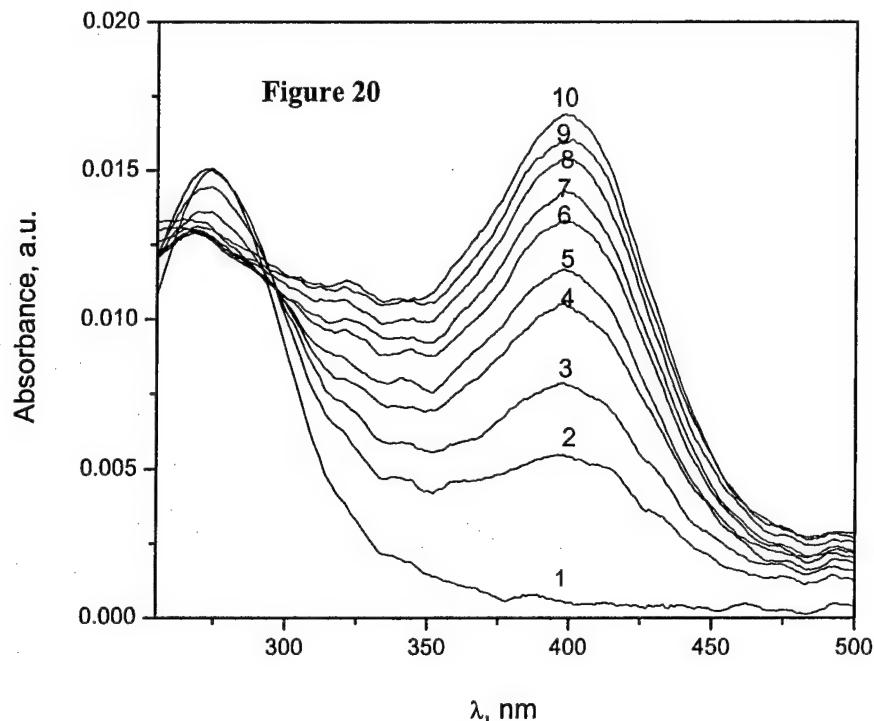


Figure 20. The UV-Vis absorption spectra of the hydrolysis product PNP at different time intervals for the detection of paraoxon solution: (1) paraoxon solution (8×10^{-7} M) and incubation time of the substrate in paraoxon solution: (2) 30 s, (3) to (10) 1, 2, 3, 4, 5, 6, 8, and 10 min.

The epifluorescence microscopy studies of the LbL adsorbed films:

The epifluorescence microscopy studies applied to the adsorbed layers provide a versatile method to visually observe the topography of these self-assembled layers and are also used to confirm the formation of aggregates. An epifluorescence microscope image taken after the fifth bilayer of CS/PTAA showed some bright orange spots, which illustrate the fluorescent CS/PTAA system (**Figure 21a**). This was compared to the epifluorescence microscope image taken after the PTAA/OPH bilayers were deposited and there was a change in the topography (**Figure 21b**) shown by the increase in size of the fluorescent orange spots.

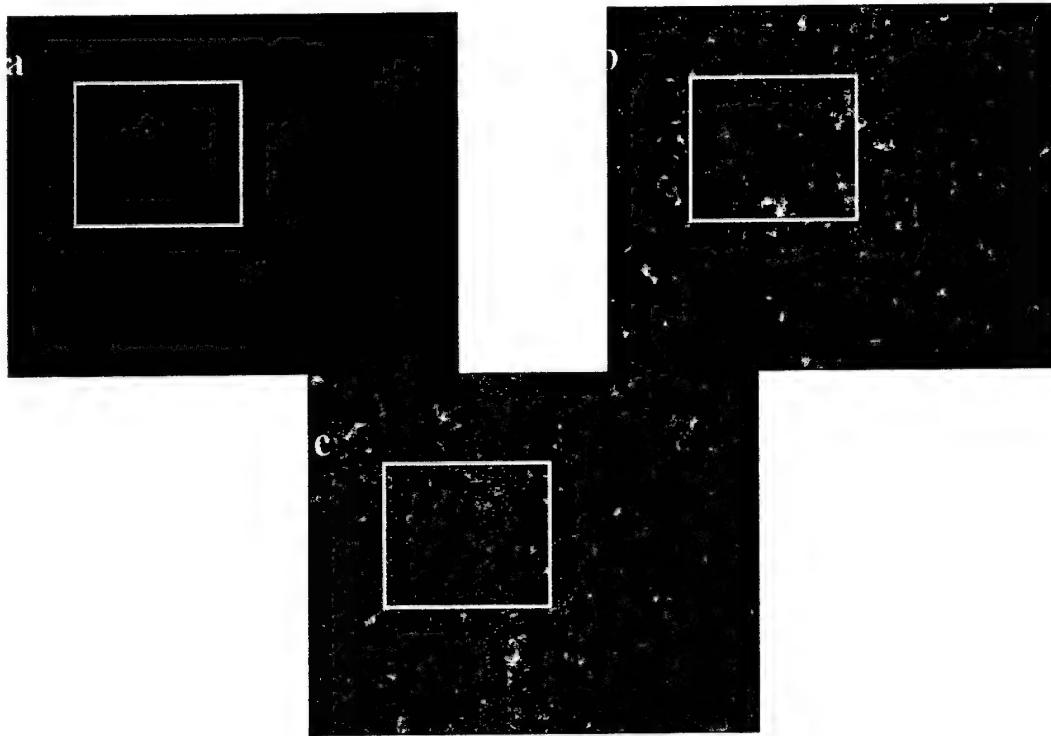


Figure 21. Epifluorescence microscope images of the bilayer system: a) 5th. bilayer CS/PTAA, b) multilayer a) plus 2-bilayers OPH/PTAA and one OPH layer, c) substrate b) after exposure to 10⁻⁹M paraoxon for 1 min. Image size, 895 μ m x 713 μ m.

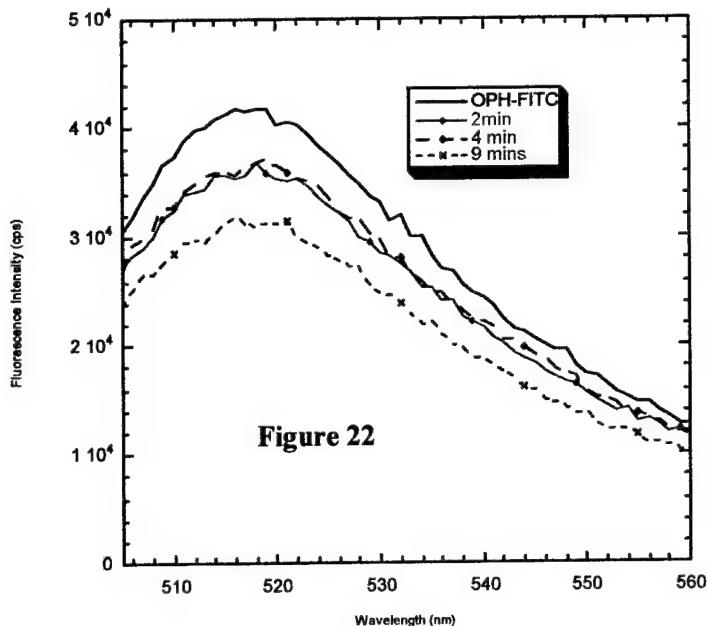
After exposure of the substrate to paraoxon solution for 1 min, the epifluorescence image was again taken and there was an additional change (Figure 21c). The intensity of the bright orange spots dramatically decreases and we attributed this fading phenomenon to the nonfluorescent PNP molecules. These results support what was illustrated from fluorescence spectroscopy. Exposure of the substrate to paraoxon causes a change in conformation of the enzymes, leading to a corresponding change in the PTAA aggregate state and hence fluorescent quenching.

From these studies, we conclude that the assembly of CS/PTAA can be successfully prepared by alternate layer-by-layer deposition on a quartz slide. This stable ultrathin film provides a well-defined substrate-independent interface for enzyme immobilization, in which the bioactivity of OPH was not compromised. This leads to fast detection of paraoxon and quick recovery times. The spectroscopic and microscopic methodologies used suggest that nonfluorescent aggregates are formed. The multilayer system has the advantage of being simple, fast and reproducible.

Surface chemistry study of OPH, OPH-FITC at the air-water interface in the presence and absence of paraoxon:

A pH 7.5 buffer was used as the subphase; the buffer was composed of 0.1 M KH_2PO_4 and 0.1 M NaOH, with 0.5 M KCl addition. It was reported that under this condition, a stable and homogenous OPH monolayer was obtained at the air-water interface, a limiting molecular area of around $7,000 \text{ \AA}^2/\text{molecule}$ was obtained. The limiting molecular area of OPH-FITC

decrease dramatically below $2,000 \text{ \AA}^2/\text{molecule}$ due to the charge carried by FITC makes the labeled protein more hydrophilic.



The fluorescence of OPH-FITC complex was measured at the air-water interface with the compression of the monolayer (Figure 22). A fluorescence emission at 520 nm was observed. It was shown that higher surface pressure resulted in fluorescence quenching of the monolayer.

The influence of the presence of paraoxon was measured by injecting $200 \mu\text{l } 10^{-3} \text{ M}$ paraoxon stock solution into the subphase beneath the monolayer (the concentration of paraoxon in the subphase is 10^{-6} M). The OPH-FITC monolayer was compressed to a surface pressure of 10 mN/m and was held constant. The fluorescence intensity of OPH-FITC dropped rapidly with paraoxon in presence in the subphase. The fluorescence quenching was caused by the interaction between the OPH at the air-water interface and paraoxon molecules in the subphase. In the presence of paraoxon, a donor-acceptor pair was formed between the fluorescent labeled OPH and paraoxon. There is energy transfer taking place between the donor-acceptor pair, the nonradiating fluorescence energy leads to quenching of the emission of the donor fluorescence, which leads in fluorescence quenching of OPH-FITC monolayer.

The LB film deposition of OPH-FITC onto quartz substrate:

At a surface pressure of 10 mN/m, the OPH-FITC was deposited onto the chemically treated hydrophobic quartz substrate. The quartz slide was held above the air-water interface and started moved vertically down at a deposition rate of 1.8 mm/min. The slide was pulled out of

the buffer when the slide reached the bottom of the trough. Nitrogen gas gently flowed the surface of the slide to let it dry. At the surface pressure of 10 mN/m the labeled OPH molecules are arranged in order at the air-water interface. The hydrophilic groups of the enzyme stay in the water while the hydrophobic groups stay in the air. Hydrophobic groups combined onto the surface of the hydrophobic quartz slide with the slide moving down and the hydrophilic groups attached to the hydrophilic groups from the first layer of the enzyme when the slide is pulling up to the air. The deposition ratio is 1.0 when the slide moves down and 0.3 when it moves up. Consequently, the hydrophilic moieties of the enzyme will interact with the paraoxon which is an ideal organization in 2-D for paraoxon detection.

The fluorescence of tests OPH-FITC LB films as a biosensor:

The fluorescence of OPH-FITC LB film was measured by fitting the slide exactly in the fluorescence cuvette. A fluorescence emission at 520 nm was observed from the labeled enzyme with the excitation wavelength of 475 nm. And the fluorescence emission spectra of the slide were shown in **Figure 23**. It was shown that the fluorescence intensity of the slide was stable after washing the surface of the slide with water and drying with nitrogen gas. The slide was then disposed in the paraoxon solution at a concentration of 10^{-6} M. After 1 minute,

the slide was taken out of the paraoxon solution and dried with nitrogen gas. A significant drop in the intensity was observed. The drop in the intensity was caused by the interaction between the enzyme and paraoxon. Enzyme-paraoxon pair results in the energy transfer from the labeled enzyme to paraoxon, which is a non-radiating energy transfer and leads to the fluorescence

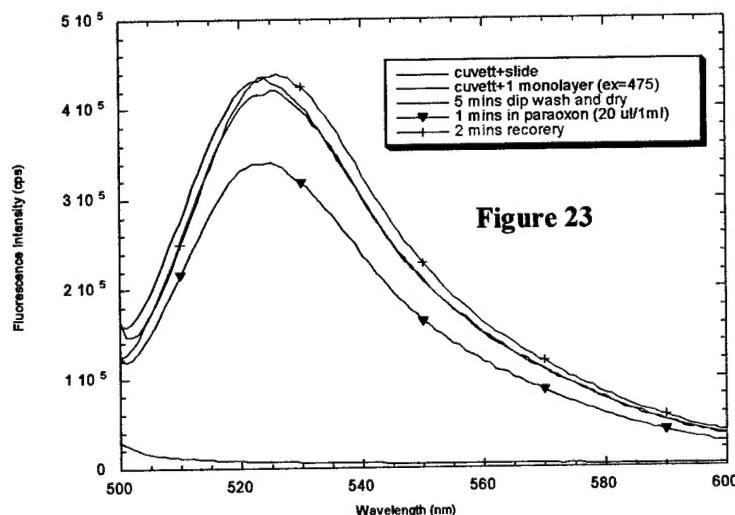


Figure 23

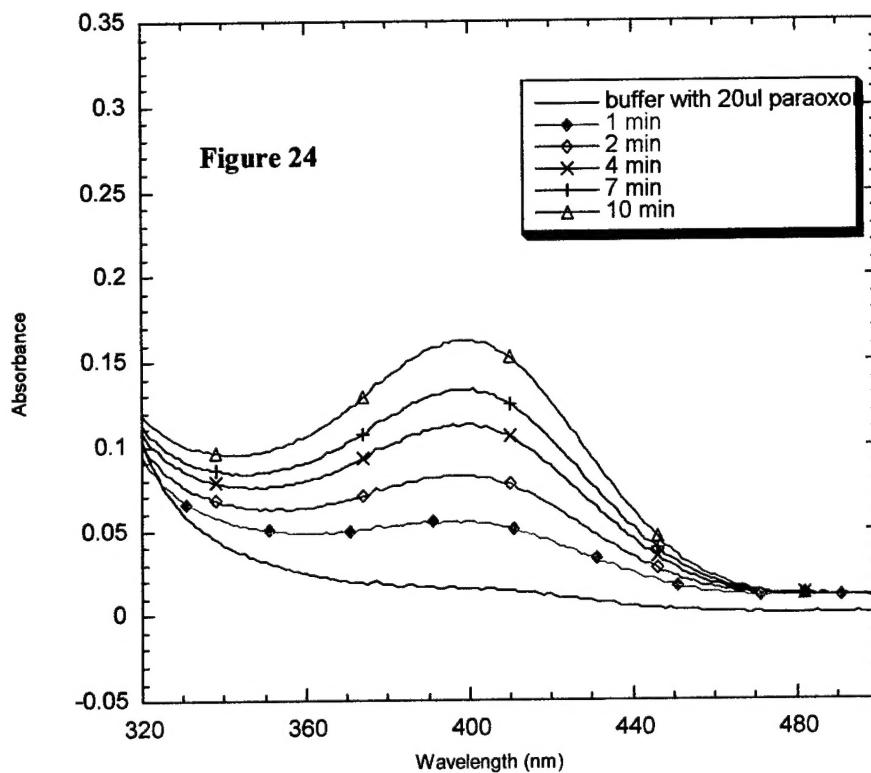
quenching of the labeled enzyme. The paraoxon hydrolysis reaction occurred at the surface of the enzyme with a high reaction rate and the enzyme-paraoxon pair can be detected instantly.

The enzyme recovery:

The enzyme can be fully regenerated and reused by thoroughly washing off the paraoxon. It was also shown in **Figure 23**, that the fluorescence intensity of the slide was fully recovered after washing off paraoxon.

The UV-Vis absorption of paraoxon degradation:

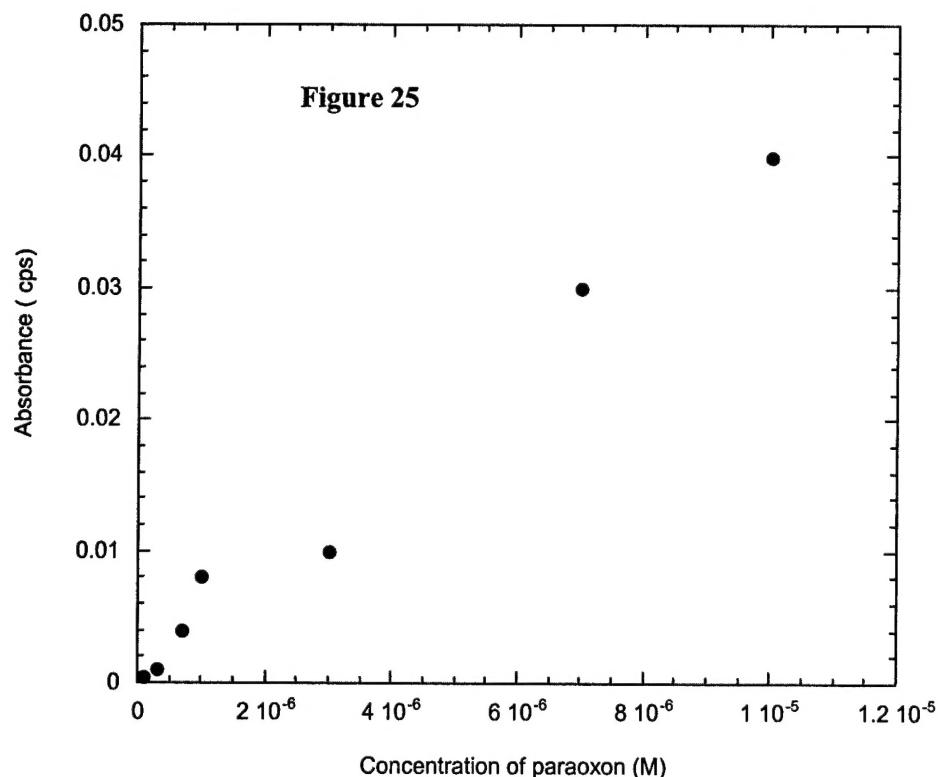
Using this biosensor, a rapid degradation of paraoxon can be observed by the UV-Vis absorption spectra (**Figure 24**). In **Figure 24**, the absorption band at 400 nm is caused by p-nitrophenol, one of the degradation products. The absorbance at 400 nm increases with the time of the LB film dipping in the paraoxon solution.



The linear relationship response between the UV-Vis signal and the concentration of paraoxon:

A linear relationship response between 10^{-7} - 10^{-5} M was observed between the UV absorbance at 400 nm and the concentration of paraoxon in presence, which is shown in **Figure 25**. The concentration of paraoxon as low as 10^{-10} M can be detected.

The linearity response between the absorbance and the concentration



Stability of the biosensor:

No great loss in the activity of the enzyme was observed for at least 3 days kept in the refrigerator.

e) Covalent linking of enzyme molecules on to the solid substrate:

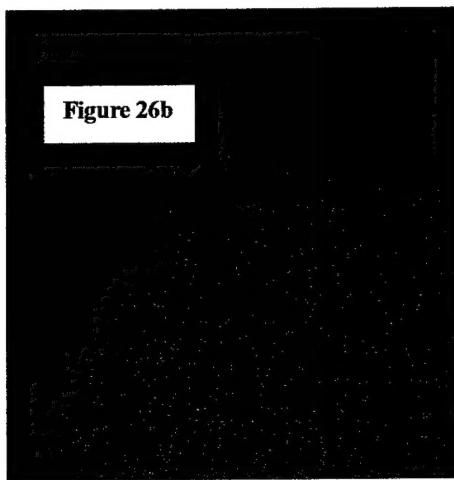
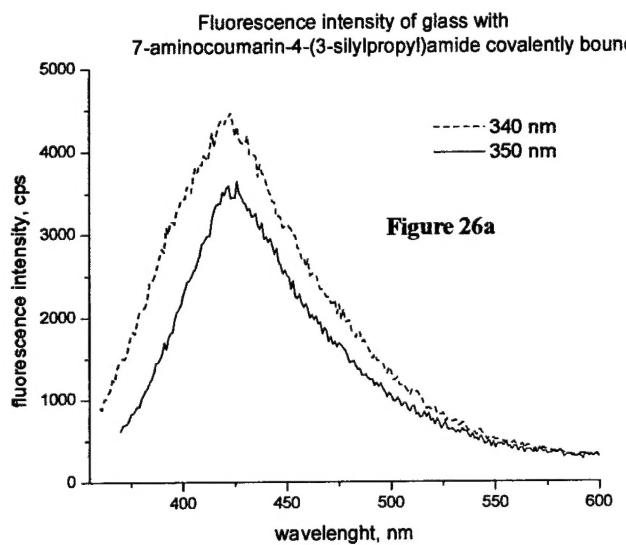
In an attempt to attach the enzyme molecules covalently to the surface of the optical fiber, we have followed the following two pathways.

We have synthesized compound **1** and compound **2**. Compound **1** (7-isothiocyanatocoumarin-4-acetic acid-3-trimethoxysilylpropylamide), can serve as a multifunctional linker that can be attached on glass surface. It has a fluorophore that can bind to the free amino functions of enzymes (Route 1). Compound **2** (methyl 7-isothiocyanatocoumarin-4-acetate) is a fluorophore-isothiocyanate that has a blue fluorescence when it is bound to enzymes (Route 2).

Route 1. The fluorophore was incorporated into the spacer. The amino function of the fluorophore (7-amino-4-acetyl-) group was converted into isothiocyanate, which readily

binds to the primary amino groups on the enzyme (Lysine-NH₂). **Route 2.** The enzyme was bound to the surface then it was labeled with the fluorophore.

We have attached the fluorophore to the silanated surface of glass slide (Cel Associates Inc. Houston, Texas) and studied the fluorescence (**Figure 26a and b**).



As part of the objectives, we have been working on developing a prototype of a fiber optic biosensor using L-B film technology. Apart from AChE, we are also working on covalent linking of OPAA and OPH on to the fiber optic surface.

f) Conclusion:

Thus, our observations clearly demonstrate that both OPAA and OPH form stable monolayers and these monolayers can be transferred on to solid substrates for developing sensors. The OPAA is highly sensitive to DFP and OPAA/FITC complex can be used for detection of DFP compound. Similarly, OPH is found to be sensitive to paraoxon and a sensor can be developed based on its sensitivity for this compound. Further, the enzyme molecules can be attached to the solid substrates covalently by using linkers and such information is highly useful for preparing a stable sensor.